RESEARCH ARTICLE

Effect of long-term vineyard monoculture on rhizosphere populations of pseudomonads carrying the antimicrobial biosynthetic genes *phlD* and/or *hcnAB*

Miroslav Svercel[1], Danilo Christen[1], Yvan Moënne-Loccoz[2,3,4], Brion Duffy[5] & Geneviève Défago[1]

[1]Plant Pathology, Institute of Integrative Biology, Zürich, Switzerland; [2]Université de Lyon, Lyon, France; [3]Université Lyon 1, Villeurbanne, France; [4]CNRS, UMR5557, Ecologie Microbienne, Villeurbanne, France; and [5]Agroscope Changins-Wädenswil, Swiss Federal Research Station for Horticulture, Plant Protection Division, Wädenswil, Switzerland

Correspondence: Geneviève Défago, Plant Pathology, Institute of Integrative Biology, ETH-Zürich, 8092 Zürich, Switzerland. Tel.: +41 44 632 3869; fax: +41 44 632 1572; e-mail: genevieve.defago@agrl.ethz.ch

Present address: Danilo Christen, Agroscope Changins-Wädenswil Research Station ACW, Centre des Fougères, CH-1964 Conthey, Switzerland.

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Abstract

The impact of repeated culture of perennial plants (i.e. in long-term monoculture) on the ecology of plant-beneficial bacteria is unknown. Here, the influence of extremely long-term monocultures of grapevine (up to 1603 years) on rhizosphere populations of fluorescent pseudomonads carrying the biosynthetic genes *phlD* for 2,4-diacetylphloroglucinol and/or *hcnAB* for hydrogen cyanide was determined. Soils from long-term and adjacent short-term monoculture vineyards (or brushland) in four regions of Switzerland were baited with grapevine or tobacco plantlets, and rhizosphere pseudomonads were studied by most probable number (MPN)-PCR. Higher numbers and percentages of $phlD^+$ and of $hcnAB^+$ rhizosphere pseudomonads were detected on using soil from long-term vineyards. On focusing on *phlD*, restriction fragment length polymorphism profiling of the last *phlD*-positive MPN wells revealed seven *phlD* alleles (three exclusively on tobacco, thereof two new ones). Higher numbers of *phlD* alleles coincided with a lower prevalence of the allele displayed by the well-studied biocontrol strain *Pseudomonas fluorescens* F113. The prevalence of this allele was 35% for tobacco in long-term monoculture soils vs. $>$ 60% in the other three cases. We conclude that soils from long-term grapevine monocultures represent an untapped resource for isolating novel biocontrol *Pseudomonas* strains when tobacco is used as bait.

Introduction

An important beneficial feature of certain long-term crop monocultures is their role in the suppression of soil-borne plant pathogens that cause root rot, crown rot, vascular wilt and damping-off diseases of many plant species (Weller *et al.*, 2002; Moënne-Loccoz & Défago, 2004; Haas & Défago, 2005; Gardener, 2007). In the case of grapevine, for instance, roots are attacked by parasitic nematodes, fungi for example *Armillaria mellea*, *Phymatotrichum omnivorum* or *Rosellinia necatrix* (Winkler, 1962), and bacteria for example *Agrobacterium tumefaciens* or *Agrobacterium vitis* (Burr *et al.*, 1998), which contribute to replant disease and therefore render replanting of grapevine in a vineyard difficult or even impossible. The facts that (1) certain sites can be replanted repeatedly and (2) old grapevines still have healthy roots suggest that the soil of these vineyards is suppressive to soil-borne diseases.

Studies of disease-suppressive soils have been limited to monocultures of several years to a few decades for obvious practical reasons, and the impact of extremely long-term monocultures (i.e. beyond many decades) on populations of key bacterial antagonists has seldom been examined, especially in the case of perennial species (Landa *et al.*, 2006). Grapevine offers a unique opportunity to study extremely long-term monocultures since vineyards dating back to pre-Roman times exist in Switzerland (Schlegel, 1973) and elsewhere in Europe. Additionally, for over a century, grapevine production has involved only a very few insect-resistant rootstock lines, on which a rich diversity of cultivars were grafted (Granett *et al.*, 2001). The fact that these extremely long-term and little diverse monocultures continue to sustain

healthy plants and expected yield points to the likely selection of a beneficial contingent of rhizosphere microorganisms.

Studies on the establishment and dynamics of soil suppressiveness to particular diseases indicate that the main factor involved is a shift in specific groups or subgroups of plant-beneficial microorganisms, rather than a change in the size or the activity of the whole microbial community (Mazzola, 2004). This selection process takes place at the plant species or even at the cultivar level (Garbeva *et al.*, 2004; Mazzola *et al.*, 2004; Okubara *et al.*, 2004; Picard *et al.*, 2004; Rotenberg *et al.*, 2007). Root-colonizing fluorescent pseudomonads that are antagonistic towards phytopathogens, and more specifically *Pseudomonas* genotypes that produce the biocontrol compounds hydrogen cyanide (HCN) and 2,4-diacetylphloroglucinol (Phl), have been repeatedly associated with disease suppressiveness of soils (Haas & Défago, 2005; Weller *et al.*, 2007).

Phl is a natural antimicrobial polyketide that is critical for the efficacy of many inoculated *Pseudomonas* biocontrol agents (Keel *et al.*, 1992; Weller *et al.*, 2002). The *phl* biosynthetic locus includes the structural gene *phlD* (Bangera & Thomashow, 1999; Paulsen *et al.*, 2005), which has been used widely as a genetic marker in population studies (McSpadden Gardener & Weller, 2001; McSpadden Gardener *et al.*, 2001; Ramette *et al.*, 2001, 2003b, 2006; Landa *et al.*, 2002a, b, 2006; Weller *et al.*, 2007). The assessment of the genotypic diversity of *phlD*$^+$ *Pseudomonas* spp. has shown a strong, although not absolute, correlation between restriction fragment length polymorphism (RFLP) of the *phlD* gene and genomic properties assayed using various PCR techniques (Mavrodi *et al.*, 2001; Ramette *et al.*, 2001).

HCN is also a biocontrol determinant in fluorescent pseudomonads (Haas & Keel, 2003; Moënne-Loccoz & Défago, 2004). It is produced by almost all Phl$^+$ strains, as well as a range of Phl$^-$ pseudomonads (Ramette *et al.*, 2003a), including biocontrol strains (Rezzonico *et al.*, 2007).

The general objective of this study was to evaluate the abundance and diversity of *phlD*$^+$ and *hcnAB*$^+$ rhizosphere pseudomonads in vineyards under long-term monoculture or in short-term counterparts, based on the hypothesis that sustainable long-term grapevine monoculture should enrich in antagonistic pseudomonads. To provide comparable rhizosphere conditions, soils from different field conditions were sampled and grown with grapevine or tobacco, a reference bait plant known to favour rhizosphere colonization by HCN$^+$ Phl$^+$ pseudomonads (Ramette *et al.*, 2003b).

Materials and methods

Survey of long-term vineyards in Switzerland

Several sites with extremely long-term monoculture vineyards (e.g. planted since the first millennium without interruption) can be found in Switzerland in official documents of the political communities or monasteries. They are located in hilly regions, and such long-standing monocultures indicate that the areas are suitable for sustainable wine production. Therefore, the whole hill is usually planted with grapevine, which renders the identification of comparable, nearby sites without a history of long-term grapevine monoculture (i.e. sites with short-term monoculture vineyards or a different land usage) very difficult. Our survey led to the identification of four such situations in different geographic regions of Switzerland. In two regions, we found (Basel and Neuchâtel) an old vineyard adjacent to a recent one (i.e. planted with grapevine since < 60 years). In another region (Valais), we found one old vineyard adjacent to a brushland, and in a fourth region (Zürich) a young vineyard was about 500 m from the old one, but both were on the same morainic layer. Because grapevine is replanted approximately every 20–25 years, it means that the old vineyards were planted 40–60 times with grapevine and the young ones one to three times. The soil parameters were similar between both sites within the same location, but differed substantially between the locations (e.g. clay content, Table 1).

Soil sampling and baiting plants

Soils were collected in November 2002 from vineyards in the four Swiss regions surveyed, i.e. Basel, Neuchâtel, Valais, Zürich and from a tobacco black root rot-suppressive soil located in Morens [soil MS8; Stutz *et al.* (1986)] (Table 1). Soil was taken at 10–30 cm depth using sterilized shovels, at each of five locations in the centre of each plot (in different rows between stocks in the vineyards). Root residues and stones were removed and soil samples from the same site were pooled. At each site, the same sampling procedure was applied on the same day to collect two composite samples (A and B). Each soil was kept at 15 °C before use. Samples A were used within 4 days after sampling (batch A) and samples B within 14 days after sampling (batch B).

Grapevine baiting plants (*Vitis riparia* × *Vitis rupestris* 3309 accession RAC 1.1) corresponding to a commonly used rootstock in Switzerland were propagated *in vitro* on ENTAV medium (Galzy, 1990). Grapevine plants were allowed an adaptation period of 12 days to build the cuticle (6 days in a covered tube, followed by 6 days in an uncovered tube) before planting in soils. Tobacco seedlings (*Nicotiana glutinosa* L.) were grown for 5 weeks in sand and fertilized with Knop's nutrient solution (Ziegler, 1983) before transplanting into soil, as described by Stutz *et al.* (1986). Grapevine or tobacco seedlings were transferred to soil-filled 300 cm^3 plastic pots with drainage holes at the bottom (one plant per pot, eight pots per batch for each soil and for each plant species). Plants were grown for a further 3 weeks and were watered as needed with distilled water to maintain the soil water

Table 1. Characteristics* of soils

Soils†,‡	Basel		Neuchâtel		Valais		Zürich		MS8 Morens
Coordinates§									
EW	610 050	610 045	553 175	553 200	580 145	580 150	703 125	702 150	559 300
SN	257 100	257 170	197 265	197 260	113 135	113 135	276 120	276 315	190 850
Grapevine monoculture in years¶	32	1603	53	1005	0	952	8	1169	0
Particle size distribution‖									
Clay (%)	41.7	36.5	24.2	26.7	13.2	13.2	24.8	20.5	14.0
Silt (%)	41.5	44.8	48.9	51.2	26.7	30.0	28.6	24.4	32.4
Sand (%)	16.8	18.7	26.9	22.1	60.1	56.8	46.6	55.1	53.6
Exchange capacity ($BaCl_2$ triethanolamine)									
Cation exchange capacity (CEC) (cmol kg^{-1})	26.4	21.5	12.5	14.4	11.5	10.6	14.4	11.9	7.1
Saturation CEC (%)	88.7	87.6	100	92.9	100	100	87.9	91.5	100
K (%)	3.4	4.1	4.3	3.1	1.2	2.6	2.9	4.0	3.0
Ca (%)	79.0	74.3	83.0	79.1	91.8	85.5	72.7	72.8	92.5
Mg (%)	5.9	8.8	12.1	9.5	6.3	11.1	11.9	14.1	3.5
Na (%)	0.4	0.4	0.6	1.2	0.7	0.7	0.5	0.6	1.0
H (%)	11.3	12.4	0	7.1	0	0	12.1	8.5	0
Soluble element									
B (hot water) (mg kg^{-1})	1.59	2.15	0.92	0.90	0.86	1.46	1.18	1.14	0.69
Reserve elements (NH_4-Ac.+EDTA 1 : 10)									
P (mg kg^{-1})	141	143	122	82	100	516	194	322	136
K (mg kg^{-1})	844	870	317	314	110	246	294	321	126
Ca (g kg^{-1})	55.1	86.5	77.3	69.3	49.8	54.0	13.6	33.5	28.9
Mg (mg kg^{-1})	540	830	710	658	486	664	432	440	230
Oligo elements (NH_4-Ac.+EDTA 1 : 10) (mg kg^{-1})									
Cu	196	290	171	156	7.9	139	149	183	4.5
Fe	518	637	443	406	508	515	606	679	367
Zn	44.5	147.4	15.4	13.2	9.9	32.1	20.1	11.5	3.9
Mn	252	143	329	325	81	102	450	372	362
Other criteria									
Organic matter (titration) (%)	1.6	4.4	1.8	1.6	3.8	2.3	2.9	1.9	4.4
pH (water)	7.9	7.5	8.0	8.0	7.8	8.0	7.4	7.7	7.6
$CaCO_3$ (total) (%)	7	29	23	19	15	18	2	8	14
N total (Kjeldahl) (%)	0.25	0.27	0.11	0.11	0.22	0.16	0.18	0.12	0.12

*Soils were analyzed by the Swiss soil testing service, Nyon (Switzerland).

†Vineyards were preselected by D. Christen (ETHZ diploma thesis 2001).

‡The crop planted before the short-term vineyards: Zürich, intensive annual cropping system; Neuchâtel, meadow; Basel, orchard. The brushland in Valais is dominated by *Prunus spinosa* with a few bushes of *Prunus triloba*.

§The National Map of Switzerland (1 : 25.000) was used for precise geographic coordinates of the plots. Two 6-number codes were used for each plot. The first code was for the east–west orientation (EW) and the second code for the south–north orientation (SN). The three first numbers of the codes correspond to the kilometers indicated in the map (every 4 cm line = 1 km). Each kilometer on the map was divided into 40 parts corresponding to the fourth and the fifth numbers of the code (every millimeter from 01 to 40). The last number of the code corresponds to a further division of the 40 parts and represents every half-millimeter (0 = full millimeter or 5 = half-millimeter).

¶The years of grapevine monoculture were identified from the following sources: Basel short (ancient photo documentation and dated plantation); Basel long (^{14}C dating – Ancient 'Centre de Recherches de Sandoz'); Neuchâtel short (document of founding of the vineyard); Neuchâtel long (documented in the 'Musée d'Art et d'Histoire' in Neuchâtel); Valais long (documented in cantonal archives in Sion), Zürich short (document of founding of the vineyard) and Zürich long (documented in archives of St Gallen).

‖Soil texture was silt loam (Basel, Neuchâtel and Zürich) or sandy loam (Valais and Morens).

content at 20% w/w. The growth chamber was set up at 22 °C, 70% relative humidity and 16 h of light (80 mE m^{-2} s^{-1}).

Quantification of rhizosphere pseudomonads

Rhizospheric pseudomonads were studied, at 3 weeks, as described by Ramette *et al.* (2003b). Briefly, the plants were removed from the pots and the root systems were shaken free of loosely adhering soil. Each root system (with closely adhering soil) was cut, weighted and transferred into a 15-mL tube containing 9.5 mL of 0.9% NaCl solution. Each tube was shaken for 40 min at 250 r.p.m., vortexed for 10 s and the sample suspension was used to inoculate (15 μL added per well) four wells of a 96-well microtitration plate

containing 135 μL of KB^{+++} medium, i.e. King's B medium (King *et al.*, 1954) supplemented with actidione (100 μg mL^{-1}), chloramphenicol (13 μg mL^{-1}) and ampicillin (40 μg mL^{-1}), for selection of fluorescent pseudomonads (Raaijmakers *et al.*, 1997). Serial 10-fold dilutions of each inoculated well were prepared in the microtitration plates, which were incubated for 3 days at 27 °C with shaking (150 r.p.m.). Bacterial growth was assessed visually and the number of pseudomonads was determined using the most probable number (MPN) technique (Alexander, 1982; Garthright & Blodgett, 2003) (see Supporting Information, Fig. S1). For storage at −80 °C, glycerol was added to all wells to achieve a final concentration of 40% and microtitration plates were sealed.

Quantification of *phlD* and *hcnAB* rhizosphere pseudomonads

The numbers of $phlD^+$ and $hcnAB^+$ pseudomonads in the rhizosphere were determined using an MPN-PCR approach (Ramette *et al.*, 2005). Briefly, 20 μL from each microtitration plate well showing bacterial growth was transferred into 150 μL of lysis solution (50 mM KCl; 0.1% Tween 20; and 10 mM Tris-HCl, pH 8.3) (Keel *et al.*, 1996) in a PCR microtitration plate (Simport Plastics, Beloeil, Canada). The suspension was centrifuged at 4025 ***g*** for 2 min and incubated for 10 min at 99 °C. The heat-lysed suspension was frozen at −20 °C for 30 min. After thawing, 4 μL of the supernatant was taken for PCR.

Amplification of *phlD* was performed using the forward primer B2BF (25-mer 5′-ACCCACCGCAGCATCGTTTATGAGC-3′) and the reverse primer BPR4 (26-mer 5′-CCGCCGGTATGGAAGATGAAAAAGTC-3′) (McSpadden Gardener *et al.*, 2001). Amplifications were carried out in 12-μL reaction mixtures containing 4 μL of lysed bacterial suspension, 1 × PCR buffer (Amersham Pharmacia, Uppsala, Sweden), bovine serum albumin (0.5 g L^{-1}; Fluka, Buchs, Switzerland), 5% dimethyl sulphoxide (Fluka), 100 μM each of dATP, dCTP, dGTP and dTTP (Amersham Pharmacia), 0.40 μM of each primer and 1.4 U of *Taq* DNA polymerase (Amersham Pharmacia). The initial denaturation (2 min at 94 °C) was followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s, with a final extension at 72 °C for 10 min. Amplification of *hcnAB* was performed using the forward primer PM2 (31-mer 5′-TGCGGCATGGGCGTGTGCCATTGCTGCCTGG-3′) and the reverse primer PM7-26R (26-mer 5′-CCGCTCTTGATCTGCAATTGCAGGCC-3′) (Svercel *et al.*, 2007), as described above. The PCR was initiated with denaturation (2 min at 94 °C) and was followed by 35 cycles of 94 °C for 30 s, 67 °C for 30 s and 72 °C for 60 s and a final extension at 72 °C for 10 min.

Primers were synthesized by MWG Biotech (Basel, Switzerland), and amplifications were performed using a PTC-100TM cycler (MJ Research Inc., Watertown, MA). The PCR products were separated in 1.5% agarose gels in 0.5 × Tris-borate-EDTA (TBE) buffer at 160 V for 1 h.

Statistics

MPN data (expressed g^{-1} of fresh root) were $\log_{10}$-transformed (Loper *et al.*, 1984) and the percentages of $phlD^+$ and $hcnAB^+$ pseudomonads were arcsine-transformed before statistical analyses. A first ANOVA was performed for data from the eight grapevine soils, using monoculture duration (i.e. long term or short term; factor 'age'), soil batch (i.e. A or B; factor 'batch'), geographic region (i.e. Basel, Neuchâtel, Valais or Zürich; factor 'location') and plant species (grapevine or tobacco; factor 'plant') as four factors, and the results are given in Table 2. For each soil batch and each plant species, further analyses of variance were then performed after inclusion of data from Morens soil MS8, i.e. for analysis of data sets encompassing the two sites for each of the four vineyard regions and Morens (nine treatments), and/or for analysis of data sets to compare each site (long-term vs. short-term vineyard) separately, whose results are displayed in Figs 1 and 2 and in Figs S2 and S3. The transformed data as well as the raw data were also subjected to a correlation analysis in relation to the actual duration of grapevine monoculture, using Pearson's correlation coefficient and Bonferroni's probability. All analyses were performed at $P < 0.05$ (and sometimes also at $P < 0.10$), using SYSTAT version 9 (SPSS Inc., Chicago, IL).

Analysis of dominant *phlD* alleles

For each dilution series, *phlD* alleles were analysed in the last *phlD*-positive MPN well (often the same dilution for different samples). Each restriction analysis was performed on 5 μL of DNA solution, using 1.5 U of either HaeIII, MspI or TaqI (Boehringer, Mannheim) over 3 h at 37 °C (HaeIII, MspI) or 60 °C (TaqI). Restriction fragments were separated by electrophoresis in ethidium bromide-stained 2.5% agarose gels, as described (Sambrook *et al.*, 1989). A 100-bp ladder (GIBCO-BRL Life Technologies Inc., Gaithersburg, MD) was used as a molecular size marker. *phlD* restriction patterns were compared with those of reference strains, according to the system of McSpadden Gardener *et al.* (2001, 2005). For each locus, the findings presented were obtained after gathering data from RFLP analyses performed from the last *phlD*-positive MPN well from two different serial dilutions per plant.

For each of the 18 location × plant × age combinations (i.e. nine combinations per plant), the following comparison criteria were computed based on *phlD* allele data. First, the sum of the different *phlD* alleles (Σ) and the dominance index of *phlD* allele K (β = occurrence of the K allele/occurrence of all alleles) were determined. Second, the

Table 2. Probability of variance distribution determined by ANOVA with the four factors age (monoculture duration), batch (soil batch), location (geographic region) and plant (plant species)

Factors	Number total pseudomonads (log CFU g^{-1} roots)	Number *phlD*$^+$ pseudomonads (log CFU g^{-1} roots)	Proportion *phlD*$^+$ pseudomonads (%)	Number *hcnAB*$^+$ pseudomonads (log CFU g^{-1} roots)	Proportion *hcnAB*$^+$ pseudomonads (%)
Age	0.476	< 0.001*	< 0.001*	0.022*	< 0.001*
Batch	< 0.001*	0.065	< 0.001*	< 0.001*	0.303
Location	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
Plant	< 0.001*	< 0.001*	< 0.001*	< 0.001*	0.001*
Batch × location	0.280	0.566	0.405	0.004*	< 0.001*
Batch × plant	< 0.001*	< 0.001*	0.639	< 0.001*	0.316
Batch × age	0.833	0.942	0.013*	0.497	0.346
Location × plant	0.102	0.014*	< 0.001*	0.109	0.012*
Location × age	0.634	0.148	0.007*	0.062	< 0.001*
Plant × age	0.286	0.026*	< 0.001*	0.002*	< 0.001*
Batch × location × plant	0.032*	0.672	0.005*	0.119	0.161
Batch × location × age	0.160	0.515	< 0.001*	0.036*	0.205
Batch × plant × age	0.057	0.707	0.013*	0.041*	0.806
Location × plant × age	0.247	< 0.001*	< 0.001*	0.027*	< 0.001*
Batch × location × plant × age	0.017*	0.010*	0.019*	0.010*	0.807

*Probability is significant at $P < 0.05$ level.

diversity of the *phlD* alleles was evaluated with regard to the number of *phlD* alleles identified (i.e. richness) using Shannon's H' index, and the distribution of *phlD* alleles among rhizosphere samples (i.e. equitability) using Shannon's E index (Shannon & Weaver, 1949). Equitability was computed from H' and the total number of different *phlD* alleles (N) as follows: $E = H'/\ln N$. In addition, the ratio of the occurrence of allele K in grapevine by that in tobacco (α) was determined for each of the nine soils. For the grapevine soils (i.e. the eight 'age' × 'location' combinations), correlation analyses were performed (as described above; $n = 8$) to investigate the relation between monoculture duration and (1) Σ, β, H' or E determined for grapevine plantlets, (2) Σ, β, H' or E determined for tobacco and (3) α (see Table 3).

Results

Effect of long-term monoculture on the total number of fluorescent pseudomonads

When grapevine or tobacco plantlets were grown in the soils collected, the total number of culturable fluorescent pseudomonads in the rhizosphere (i.e. 10^8–10^9 g^{-1} root) depended significantly on the soil batch, the geographic region and the plant species, but not on whether monoculture was short or long (Table 2, Figs 1 and 2, Figs S2 and S3). Indeed, there was no significant correlation (not shown) between the actual duration of grapevine monoculture and the total number of fluorescent pseudomonads in the rhizosphere (Figs S2 and S3).

Effect of long-term monoculture on the numbers of *phlD* and *hcnAB* fluorescent pseudomonads

Based on MPN data, *phlD*$^+$ and *hcnAB*$^+$ pseudomonads represented, respectively, 5–57% and 17–47% of all culturable fluorescent pseudomonads in the rhizosphere for both grapevine and tobacco plantlets (Figs 1 and 2). Whether the soil used to grow plantlets originated from short- or long-term grapevine monoculture sites had a statistically significant impact on both the number and the percentage of rhizosphere *phlD*$^+$ pseudomonads, as well as on both the number and the percentage of rhizosphere *hcnAB*$^+$ pseudomonads (Table 2). Indeed, soils with a long-term monoculture history were associated with higher values for the number (6.0×10^7 vs. 2.3×10^7 g^{-1} root) and the percentage (23% vs. 13%) of rhizosphere *phlD*$^+$ pseudomonads, and the number (7.7×10^7 vs. 3.2×10^7 g^{-1} root) and the percentage (32% vs. 26%) of rhizosphere *hcnAB*$^+$ pseudomonads (Figs 1 and 2). ANOVA indicated that the results also depended on the geographic region and the plant species (for all four variables) and to a lesser extent on the soil batch (only for the percentage of *phlD*$^+$ pseudomonads and the number of *hcnAB*$^+$ pseudomonads) (Table 2).

When only results from the grapevine plantlet rhizosphere were considered, each older vineyard soil yielded an apparently higher percentage of rhizosphere *phlD*$^+$ pseudomonads compared with its younger counterpart (Fig. 1), and the difference was statistically significant in seven of eight cases (in the regions Basel, Neuchâtel and Valais with soil batch A, vs. in all four vineyard locations with batch B).

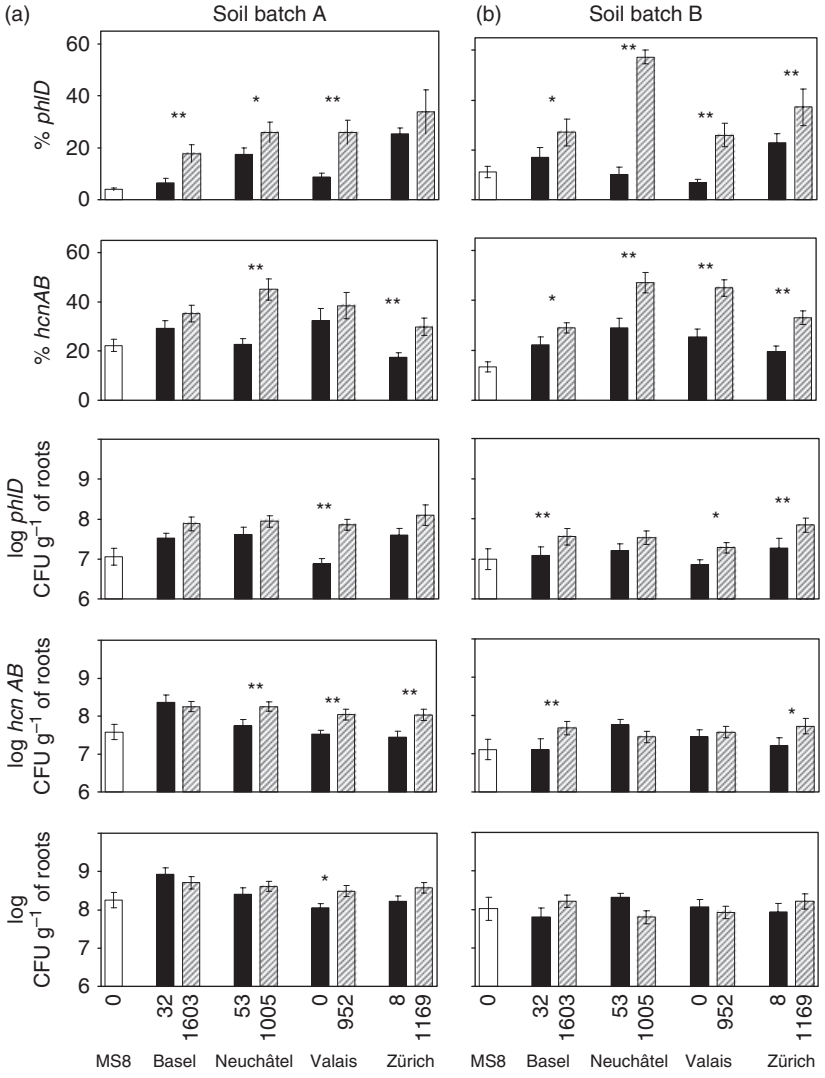

Fig. 1. Differences between young vineyards (black bars), old vineyards (grey bars) and crop rotation soil (white bars; MS8) in terms of the frequencies of $phlD^+$ and $hcnAB^+$ pseudomonads, and the numbers of $phlD^+$, $hcnAB^+$ and total pseudomonads obtained from grapevine roots. The numbers above region names represent the duration of grapevine monoculture. The soil batches A and B represent duplicated experiments. ANOVA was performed on data sets encompassing the two sites for each of the four vineyard regions (separate comparison of long-term vs. short-term vineyard). For each soil batch, data are shown as means ± SE ($n = 8$) and significant differences ($P < 0.1$; 0.05) are indicated by asterisks (*; **). The effect of the duration of grapevine monocultures on pseudomonad populations is shown in Fig. S2.

Similarly, the number of $phlD^+$ pseudomonads was higher in old monocultures and differed statistically in four of eight cases (in the region Valais with soil batch A, and in the regions Basel, Valais and Zürich with batch B). Similarly, the percentage of rhizosphere $hcnAB^+$ pseudomonads was seemingly higher for older vineyard soils (in most cases), and the differences were statistically significant in six of the eight individual old/young comparisons (Fig. 1). Concerning the number of $hcnAB^+$ pseudomonads, the rhizosphere populations were significantly larger in five of eight cases in long-term monoculture soils compared with the short-term counterparts (Fig. 1). When data were assessed based only on the actual duration of grapevine monoculture, without considering the four regions, longer durations did not consistently translate into increased numbers and percentages of $phlD^+$ pseudomonads and of $hcnAB^+$ pseudomonads in plantlet rhizospheres (Fig. S2). However, a significantly positive correlation was found with the number of rhizosphere $phlD^+$ pseudomonads in soil batches A ($r = 0.76$, $n = 9$ and $P = 0.017$) and B ($r = 0.82$, $n = 9$ and $P = 0.007$) and the number of rhizosphere $hcnAB^+$ pseudomonads in soil batch A only ($r = 0.88$, $n = 9$ and $P = 0.002$).

When tobacco was used as bait, the numbers of $phlD^+$, $hcnAB^+$ and total culturable pseudomonads in the rhizosphere were often lower (by 0.5–1.5 log g^{-1} root) in comparison with grapevine data (Figs 1 and 2). In many cases, the percentages of $phlD^+$ and $hcnAB^+$ pseudomonads were less on tobacco than on grapevine. The main exception was Morens, a soil without a grapevine history, where the percentages of $phlD^+$ and of $hcnAB^+$ pseudomonads in the rhizosphere were higher with tobacco than with grapevine.

When the effect of long-term grapevine monoculture was assessed based only on results from the tobacco rhizosphere, the trend was similar as to that of grapevine plants in the case of the number and percentage of $phlD^+$ pseudomonads when using soil from the three regions (Basel, Neuchâtel and

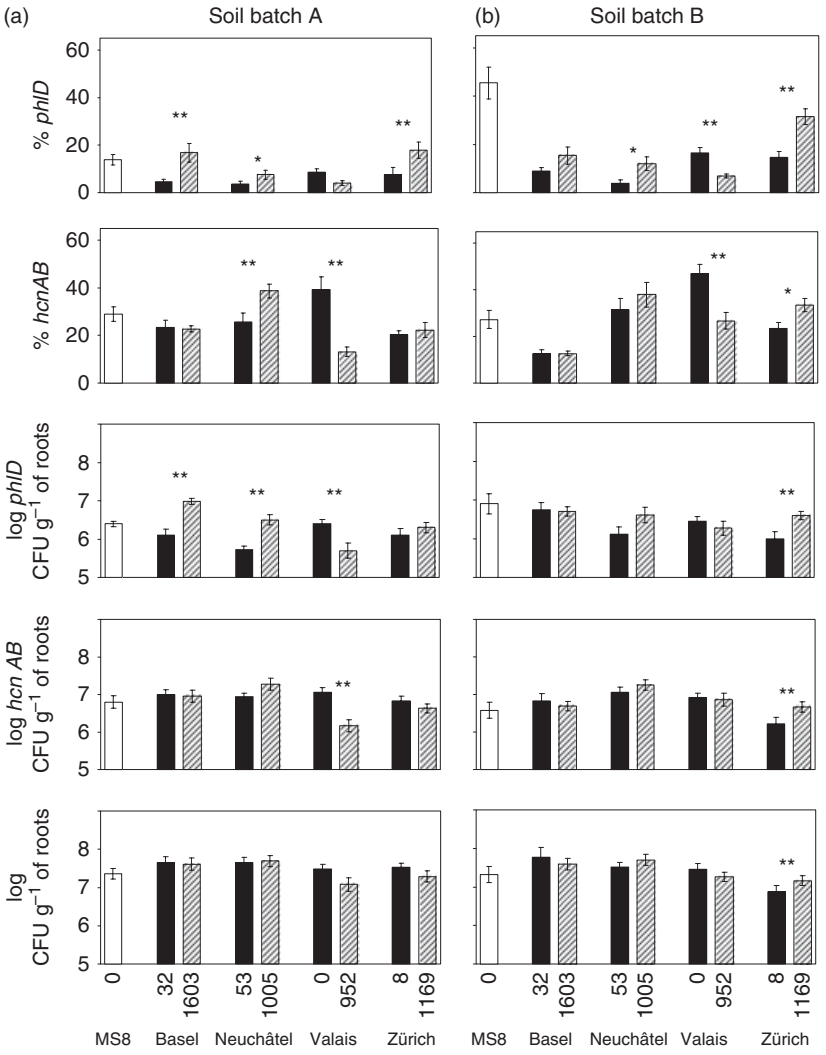

Fig. 2. Differences between young vineyards (black bars), old vineyards (grey bars) and crop rotation soil (white bars; MS8) in terms of the frequencies of $phlD^+$ and $hcnAB^+$ pseudomonads, and the numbers of $phlD^+$, $hcnAB^+$ and total pseudomonads obtained from tobacco roots. The numbers above region names represent the duration of grapevine monoculture. ANOVA was performed on data sets encompassing the two sites for each of the four vineyard regions (separate comparison for long-term vs. short-term vineyard). The soil batches A and B represent duplicated experiments. For each soil batch, data are shown as means ± SE ($n = 8$) and significant differences ($P < 0.1$; 0.05) are indicated by asterisks (*; **). The effect of the duration of grapevine monocultures on pseudomonad populations is shown in Fig. S3.

Zürich) where the rhizosphere levels were higher in long-term than in short-term vineyard soils (Fig. 2). The trend was statistically significant in eight of the 12 individual old/young comparisons. There was a different situation in these three regions in terms of the number and percentage of $hcnAB^+$ pseudomonads, where the values were significantly higher in just three of the 12 individual old/young comparisons. Unlike with soils from these three regions, growing tobacco in the old Valais vineyard soil yielded lower numbers of $phlD^+$ and of $hcnAB^+$ pseudomonads (for soil batch A), as well as lower percentages of $phlD^+$ pseudomonads (for soil batch B) and $hcnAB^+$ pseudomonads (for both soil batches).

Diversity of the *phlD* gene

RFLP patterns of *phlD* amplicons from the last *phlD*-positive dilution wells (i.e. of dominant *phlD* alleles) were assessed by summing band sizes (to determine the number of alleles), and by comparison with 58 reference strains from a worldwide collection (McSpadden Gardener *et al.*, 2000, 2005; Wang *et al.*, 2001). For the 288 plants (tobacco and grapevine), only one allele was found, except that 11 of the 144 tobacco plants revealed two *phlD* alleles (4% from the total number of plants tested). When two alleles were identified in the same well, one typically yielding a less intense band, they were also identified (this time alone) for another plant grown in the same soil.

Five previously known *phlD* alleles i.e. A (documented in strains CHA0 and Pf-5), D (strains C*1A1 and Q8R1-96), F (strains JMP6 and JMP6R), K (strain F113) and M (strains PITR2 and D27B1) and two new alleles (designated U and V) were detected. Screening of laboratory collections enabled identification of allele U in strain Q37-87, which was previously characterized as rep-PCR genotype E (McSpadden Gardener *et al.*, 2000), but that yielded a different profile

Table 3. Occurrence of *phlD* alleles, as indicated by the number of plants from which the alleles were found*

Treatment	Years of grapevine monoculture	*phlD* alleles							Σ†	α‡	β§	*H′*¶	*E*‖
		A	D	F	K	M	U	V					
Grapevine													
Morens MS8	0		1	8	5				3	0.6	0.36	0.87	0.80
Basel	32		2	6	5				3	1.0	0.39	1.00	0.91
	1603			4	9				2	3.0	0.69	0.62	0.89
Neuchâtel	53	2	2		12				3	1.1	0.75	0.74	0.67
	1005				15				1	5.0	1.00	0.00	0.00
Valais	0				16				1	1.0	1.00	0.00	0.00
	952				16				1	1.5	1.00	0.00	0.00
Zürich	8				14				1	2.3	1.00	0.00	0.00
	1169	1	2	1	9				4	4.5	0.69	0.94	0.68
Tobacco													
Morens MS8	0	1		5	8				3		0.57	0.87	0.80
Basel	32	4		5	5	1			4		0.33	1.27	0.92
	1603	1	3	3	3	1	2		6		0.23	1.70	0.95
Neuchâtel	53	2	1	1	11				4		0.73	0.86	0.62
	1005	1		1	3	3	3	1	6		0.25	1.65	0.91
Valais	0				16				1		1.00	0.00	0.00
	952	1		3	11				3		0.73	0.74	0.67
Zürich	8	1	1	2	6	1	2	1	7		0.43	1.66	0.85
	1169	2	2	1	2	1	1	3	7		0.17	1.83	0.94
Number of plants		15	14	40	166	7	8	5					

*Combined results obtained when studying soil batches A and B. For each dilution series, *phlD* alleles were analyzed in the last *phlD*-positive MPN well.
†Sum of different *phlD* alleles.
‡Ratio of the occurrence of allele K in grapevine/occurrence of allele K in tobacco.
§Dominance index of *phlD* allele K (= occurrence of allele K/occurrence of all alleles).
¶Shannon's richness index.
‖Equitability = $H'/\ln N$, with N = number of alleles.

here with HaeIII, and allele V in strain K93.2. Four alleles were obtained on both grapevine and tobacco (A, D, F and K) and the three others (M, U and V) exclusively on tobacco (Table 3). The most frequent allele was K, which was found in all treatments, followed by alleles F and A, detected in $>$ 50% of the MPN wells studied.

When grapevine was used as bait, the number of *phlD* alleles in long-term vineyard soils was higher (Zürich, four vs. one allele), identical (Valais, one allele) or lower (Basel, two vs. three alleles; Neuchâtel, one vs. three) than in the short-term counterparts and this paralleled the prevalence of allele K (i.e. higher when the allele number was lower) (Table 3). When tobacco was used, the number of *phlD* alleles in long-term vineyard soils was higher (Valais, three vs. one allele; Basel, six vs. four; and Neuchâtel, six vs. four, as allele K was less frequent) or identical (Zürich, seven alleles) in comparison with the short-term counterparts. Overall, allele K was found with 85% of grapevine plantlets and 35% of tobacco plantlets in long-term monoculture soils, vs., respectively, 78% and 62% in the short-term counterparts. The presence or absence of allele K in these rhizosphere samples was confirmed on testing other (randomly selected) MPN wells corresponding to lower *phlD*-positive dilutions (not shown). In addition, the ratio between grapevine and tobacco for allele K was 50% higher or more for plants grown in long-term monoculture soils vs. short-term counterparts in the four regions. Shannon's H' index was identical or lower when comparing grapevine vs. tobacco baiting plants (Table 3). However, H' was essentially similar for long-term monoculture soils and short-term counterparts, regardless of whether grapevine or tobacco was considered. In addition, there was no significant correlation between monoculture duration and (1) Σ, β, H' or E determined for grapevine plantlets, (2) Σ, β, H' or E determined for tobacco and (3) α.

Discussion

Grapevine is a perennial plant, and each year the roots, leaves and small branches are left to decompose and contribute to the organic matter status of the top soil (i.e. the soil layer that was sampled in this study). In addition, root exudates and root architecture modify soil quality and structure. Despite differences in the soil composition, microclimatic conditions, grapevine cultivars and plot management from one region to the other, it is expected that

these factors had cumulated and developed to a much higher extent in long-term grapevine monoculture in comparison with short-term grapevine monoculture. In this context, four pairs of sites were selected for the current work. It is important to note that two major crop management features have been implemented over the last century, i.e. the exclusive use of a few foreign rootstocks for insect resistance (Granett *et al.*, 2001) and yearly treatments with copper sulphate against fungal pathogens. This concerned all current sites, except the brushland in Valais and soil MS8 in Morens, as illustrated by much lower soil copper contents (Table 1).

This work was based on the hypothesis that sustainable, long-term grapevine monoculture should lead to an enrichment in antagonistic pseudomonads, and indeed statistical analyses indicated that the rhizosphere of plantlets grown in long-term grapevine monoculture soils displayed a higher number and percentage of *phlD*$^+$ pseudomonads, as well as a higher number and percentage of *hcnAB*$^+$ pseudomonads, but it had no significant impact on the total number of rhizosphere pseudomonads. Similar findings were obtained when grapevine plantlet data were analysed separately, one soil batch at a time, but the statistical power was less and the differences were statistically significant for most, but not all pair of soils. Interestingly, when tobacco was used as bait, the percentages of rhizosphere *phlD*$^+$ pseudomonads remained higher in long-term monoculture soils in comparison with neighbouring short-term vineyard soils. *phlD*$^+$ pseudomonads from laboratory collections are also HCN$^+$, except one strain (Wang *et al.*, 2001), in which the *hcnABC* locus is inactivated (Rezzonico *et al.*, 2007). Here, the *hcnAB*$^+$ population was higher than the *phlD*$^+$ population, pointing to the presence of *phlD*$^+$ *hcnAB*$^+$ pseudomonads as well as *phlD*$^-$ *hcnAB*$^+$ strains. In accordance with the current results, the *phlD*$^+$ population was higher in soil after a century of wheat and/or flax monoculture than under crop rotation (Landa *et al.*, 2006). In addition, the percentage of rhizosphere *phlD*$^+$ pseudomonads was higher in a soil suppressive to black root rot than in a conducive soil (Ramette *et al.*, 2003b).

Morens soil MS8 was included in the study as a reference, because the tobacco rhizosphere is known to select Phl$^+$ HCN$^+$ pseudomonads in this soil (Ramette *et al.*, 2003b). The current results indicate that the enrichment of *phlD*$^+$ and/or *hcnAB*$^+$ pseudomonads in the grapevine rhizosphere was higher in grapevine soils (especially under long-term monoculture) compared with Morens soil, but this was not the case in the tobacco rhizosphere. On the one hand, in Morens soil, which was probably never planted with grapevine, tobacco plantlets baited a similar or a higher percentage of *phlD*$^+$ pseudomonads (but not HCN$^+$ pseudomonads) than did grapevine plantlets. On the other hand, when we baited long-term monoculture grapevine soils with tobacco (a plant not present in the vineyards, but that grew well in these soils), the percentage of *phlD*$^+$ pseudomonads (but not HCN$^+$ pseudomonads) was lower than when grapevine plantlets were used. These results suggest that grapevine monoculture has favoured grapevine-adapted *phlD*$^+$ pseudomonads, and this hypothesis was assessed by studying *phlD* alleles.

Four dominant *phlD* alleles were recovered from the grapevine rhizosphere vs. as many as seven from tobacco. This latter number is high, as Landa *et al.* (2002b) and McSpadden Gardener *et al.* (2000) found a maximum of four *phlD* alleles per field sample using the same 629-bp *phlD* amplicon as in the present study (vs. up to four alleles with a 745-bp *phlD* amplicon; Ramette *et al.*, 2001). Here, the lower number of dominant *phlD* alleles on grapevine was explained by a higher prevalence of allele K, and up to 85% of grapevine plantlets displayed this allele in long-term vineyard monoculture soils. Therefore, *phlD*$^+$ pseudomonads less competitive for rhizosphere colonization of grapevine plantlets than those displaying *phlD* allele K managed to survive as soil saprophytes and could be baited by tobacco. Moreover, the long-term monoculture soils maintained a higher number of *phlD* alleles (revealed after baiting by tobacco plants) than the short-term monoculture soils in the three sites where both types of soils are located side by side (i.e. Basel, Neuchâtel and Valais). Allele K is found in the well-studied biocontrol strain *Pseudomonas fluorescens* F113, and interestingly F113-like pseudomonads were readily evidenced in the strawberry rhizosphere by PCR-denaturing gradient gel electrophoresis targeting the 16S rRNA gene (Costa *et al.*, 2006) or gacA (Costa *et al.*, 2007). Picard & Bosco (2006) found that maize hybrids and their parents selected different *phlD* alleles, and distinct genotypes of *phlD*$^+$ pseudomonads were enriched during monoculture, whereas only one *phlD* allele had been detected under crop rotation (Landa *et al.*, 2006; Weller *et al.*, 2007). The larger number of distinct alleles in the current case compared with long-term monocultures of annual plants such as wheat and flax (Landa *et al.*, 2006) is perhaps due to a lower level of anthropogenic soil disturbance with perennial plant grapevine.

Soil properties varied more between regions than between adjacent vineyards. Abiotic factors (Ownley *et al.*, 2003) and soil type (Latour *et al.*, 1996; Dalmastri *et al.*, 1999; Marschner *et al.*, 2001) are known to influence the composition of microbial populations and the production of biocontrol metabolites important for microbial survival (Duffy & Défago, 1999; Haas & Keel, 2003). Here, it remains to be examined as to how soil properties could also have influenced the selection of specific *Pseudomonas* genotypes, similar to the observation that zinc and certain other microelements can select for mutant subpopulations in pseudomonads (Duffy & Défago, 2000).

In conclusion, we have identified a positive effect of long-term grapevine monoculture on the number and percentage of rhizosphere-colonizing pseudomonads carrying the biocontrol genes *phlD*$^+$ and/or *hcnAB*$^+$. *Pseudomonas* strains displaying allele K were highly prevalent in the grapevine rhizosphere (i.e. $>$ 60%), regardless of the soil, but in long-term grapevine monoculture soils, they were in a minority in the tobacco rhizosphere (but not in the grapevine rhizosphere). A lower prevalence of allele K coincided with higher numbers of the other dominant *phlD* alleles. This means that tobacco was useful to recover alternative *phlD*$^+$ genotypes, which are less adapted to grapevine. We conclude that long-term grapevine monoculture soils represent an untapped resource for isolating new *Pseudomonas* strains with potential biocontrol activity, when tobacco is used to bait the rhizosphere pseudomonads.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Most probable number–PCR (MPN-PCR) approach to determine the population structure of rhizosphere colonizing pseudomonads with biocontrol relevant genes.
Fig. S2. Effect of duration of grapevine monocultures on the numbers and frequencies of *phlD*$^+$ or *hcnAB*$^+$ pseudomonads and on the total pseudomonad population obtained from roots of grapevine grown in vineyard soils and in nonvineyard soils (brushland adjacent to a 952-years-old vineyard in Valais, and tobacco disease suppressive soil from Morens).
Fig. S3. Effect of duration of grapevine monocultures on the numbers and frequencies of *phlD*$^+$ or *hcnAB*$^+$ pseudomonads and on the total pseudomonad population obtained from roots of tobacco grown in vineyard soils and in nonvineyard soils (brushland adjacent to a 952-years-old vineyard in Valais, and tobacco disease suppressive soil from Morens).

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